Detailed Description of the Invention

This invention provides a method for inhibiting fusion of HIV-1 to $CD4^+$ cells which comprises contacting CD4 cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited.

This invention also provides a method for inhibiting HIV-1 infection of $CD4^+$ cells which comprises contacting $CD4^+$ cells with a non-chemokine agent capable of binding to a chemokine receptor in an amount and under conditions such that fusion of HIV-1 to the $CD4^+$ cells is inhibited, thereby inhibiting the HIV-1 infection.

In this invention, a chemokine means RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. A chemokine receptor means a receptor capable of binding RANTES, MIP-1-α, MIP-1-β or another chemokine which blocks HIV-1 infection. Such chemokine receptor includes but not limited to CCR5, CXCR4, CCR3 and CCR-2b.

Throughout this application, the receptor "fusin" is also named CXCR4 and the chemokine receptor C-C CKR5 is also named CCR5.

The HIV-1 used in this application unless specified will mean clinical or primary or field isolates or HIV-1 viruses which maintain their clinical characteristics. The HIV-1 clinical isolates may be passaged in primary peripheral blood mononuclear cells. The HIV-1 clinical isolates may be macrophage-trophic.

The non-chemokine agents of this invention are capable of binding to chemokine receptors and inhibiting fusion of HIV-1 to $CD4^+$ cells. The non-chemokine agents include, but are not limited to, chemokine fragments and chemokine derivatives and analogues, but do not include naturally occurring chemokines. The non-chemokine agents include

multimeric forms of the chemokine fragments and chemokine derivatives and analogues or fusion molecules which contain chemokine fragments, derivatives and analogues linked to other molecules.

The non-chemokine agents do not include bicyclams and their derivatives as described in U.S.Patent No. 5,021,409, issued June 4, 1991, the content of which is incorporated by reference into this application. Some bicyclam derivatives have been previously described with antiviral activities (15, 16).

In an embodiment of this invention, the non-chemokine agent is an oligopeptide. In another embodiment, the non-chemokine agent is a polypeptide. In still another embodiment, the non-chemokine agent is an antibody or a portion thereof. Antibodies against the chemokine receptor may easily be generated by routine experiments. It is also within the level of ordinary skill to synthesize fragments of the antibody capable of binding to the chemokine receptor. In a further embodiment, the non-chemokine agent is a nonpeptidyl agent.

Non-chemokine agents which are purely peptidyl in composition can be either chemically synthesized by solid-phase methods (Merrifield, 1966) or produced using recombinant technology in either prokaryotic or eukaryotic systems. The synthetic and recombinant methods are well known in the art.

Non-chemokine agents which contain biotin or other nonpeptidyl groups can be prepared by chemical modification of synthetic or recombinant chemokines or non-chemokine agents. One chemical modification method involves periodate oxidation of the 2-amino alcohol present on chemokines or non-chemokine agents possessing serine or threonine as their N-terminal amino acid (Geophegan and Stroh, 1992). The resulting aldehyde group can be used to link peptidyl or

HIV-1 to CD4$^+$ cells with the proviso that the agent is not a known bicyclam or its known derivatives. In an embodiment, the non-chemokine is a polypeptide. In a further embodiment, this polypeptide is a fragment of the chemokine RANTES (Gong et al., 1996). In a still further embodiment, the polypeptide may also comprise the RANTES sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first eight N-terminal amino acids of the RANTES sequence (SEQ ID NO:5).

In a separate embodiment, the polypeptide may comprise the MIP-1ß sequence with deletion of the N-terminal amino acids of said sequence. The deletion may be the first seven, eight, nine or ten N-terminal amino acids of the MIP-1ß sequence.

In another embodiment of non-chemokine agent, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by addition of an amino acid or oligopeptide. In a separate embodiment, the polypeptide comprises the MIP-1ß sequence with the N-terminal sequence modified by removing the N-terminal alanine and replaced it by serine or threonine and additional amino acid or oligopeptide or nonpeptidyl moiety. In a further embodiment, the additional amino acid is methionine.

As described infra in the section of Experimental Details, a cofactor for HIV-1 fusion and entry was identified and designated "fusin" (Feng et al., 1996). This invention provides an agent which is capable of binding to fusin and inhibiting infection. In an embodiment, the agent is an oligopeptide. In another embodiment, the agent is an polypeptide.

In a further embodiment, the polypeptide comprises SDF-1 with deletion of the N-terminal amino acids of said sequence. The deletion may be the first six, seven, eight, or nine N-terminal amino acids of the SDF-1 sequence.

This invention also provides the above non-chemokine agent, wherein the polypeptide comprises SDF-1 sequence with the N-terminal sequence modified to produce antagonistic effect to SDF-1. One modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with biotin. Another modification is to replace the N-terminal glycine of SDF-1 by serine and derivatized with methionine. A further modification is to add the N-terminus of SDF-1 with a methionine before the terminal glycine.

In still another embodiment, the agent is an antibody or a portion of an antibody. In a separate embodiment, the agent is a nonpeptidyl agent.

The agents capable of binding to fusin may be identified by screening different compounds for their capability to bind to fusin in vitro.

A suitable method has been described by Fowlkes, et al. (1994), international application number: PCT/US94/03143, international publication number: WO 94/23025, the content of which is incorporated by reference into this application. Briefly, yeast cells having a pheromone system are engineered to express a heterologous surrogate of a yeast pheromone system protein. The surrogate incorporates fusin and under some conditions performs in the pheromone system of the yeast cell a function naturally performed by the corresponding yeast pheromone system protein. Such yeast cells are also engineered to express a library of peptides whereby a yeast cell containing a peptide which binds fusin exhibits modulation of the interaction of surrogate yeast pheromone system protein with the yeast pheromone system and this modulation is a selectable or screenable event. Similar approaches may be used to identify agents capable of binding to both fusin and the chemokine receptor C-C CKR-5.

This invention also provides pharmaceutical compositions comprising an amount of such non-chemokine agents or agents

Experimental Details

FIRST SERIES OF EXPERIMENTS

1) Chemokines inhibit fusion mediated by the envelope glycoprotein from a macrophage-tropic primary isolate of HIV-1 but not from a laboratory-adapted T-lymphotrophic strain of the virus

The chemokines RANTES, MIP-1α and MIP-1β were obtained from R & D systems (Minneapolis, MN). They were tested in the RET assay for ability to inhibit fusion between HeLa-env_{JR-FL} cells (expressing gp120/gp41 from the macrophage tropic isolate HIV-1_{JR-FL}) and PM1 cells, or for inhibition of fusion between HeLa-env_{LAI} cells (expressing gp120/gp41 from the laboratory-adapted strain HIV-1_{LAI}) and various $CD4^+$ T lymphocyte cell lines. As shown in Figure 1, all three chemokines inhibited fusion mediated by the macrophage tropic virus envelope glycoprotein, but not that mediated by the laboratory-adapted strain envelope glycoprotein.

The ability of the chemokines to block the interaction between CD4 and HIV-1 gp120 which occurs at virus attachment was then tested. It was found that the chemokines did not inhibit this interaction (Figure 2), demonstrating that their blockade of HIV-1 envelope glycoprotein-mediated membrane fusion occurs at the membrane fusion event itself, rather than the initial CD4-gp120 interaction which precedes fusion.

2) Non-chemokine peptides and derivatives that inhibit HIV-1 fusion

The non-chemokines include chemokine fragments and chemokine derivatives that are tested in the RET assay to determine which are active in inhibiting HIV-1 membrane fusion. Particular attention is focused on fragments or derivatives that inhibit HIV-1 fusion but do not activate leukocyte responses. These non-chemokines include:

Table 1: Inhibition of HIV-1 entry in PM1 cells and CD4+ T-cells by ß-chemokines

% luciferase activity

	BaL	ADA	NL4/3	HxB2	MuLV
a) PM1 cells					
control without virus	2	2	2	5	3
control with virus	100	100	100	100	100
+R/Mα/Mß (50/50/50)	2	3	92	117	100
+RANTES (100)	1	1	nd	nd	nd
+MIP-1α(100)	54	54	nd	nd	nd
+MIP-1ß (100)	1	6	nd	nd	nd
+MCP-1 (100)	46	50	nd	nd	nd
+MCP-2 (100)	28	26	nd	nd	nd
+MCP-3 (100)	58	46	nd	nd	nd
b)	**JR-FL**	**HxB2**	**MuLV**		
LW4 CD4+ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	14	68	nd		
LW5 CD4+ T-cells					
control without virus	1	1	1		
control with virus	100	100	100		
+R/Mα/Mß (200/200/200)	15	73	nd		

Table 1 legend:

PM1 cells were cultured as described by Lusso et al (12). Ficoll/hypaque-isolated PBMC from laboratory workers (LW) stimulated with PHA for 72h before depletion of CD8+ Lymphocytes with anti-CD8 immunomagnetic beads (DYNAL, Great Neck, NY). CD4+ Lymphocytes were maintained in culture medium containing interleukin-2 (100U/ml; Hofmann LaRoche, Nutley, NJ), as described previously (3). Target cells (1-2x10^5) were infected with supernatants (10-50ng of HIV-1 p24) from 293-cells co-transfected with an NL4/3Δenv-luciferase vector and a HIV-1 env-expressing vector (10,11). ß-Chemokines (R & D Systems, Minneapolis) were added to the target cells simultaneously with virus, at the final concentrations (ng/ml) indicated in parentheses in the first column. The ß-chemokine concentration range was selected based on prior studies (2,3). After 2h, the cells were washed twice with PBS, resuspended in ß-chemokine-containing media and maintained for 48-96h. Luciferase activity in

Table 3: C-C CKR-5 expression permits infection of CD4-expressing cells by primary, NSI HIV-1 strains

									R/Mα/Mß
		pcDNA3.1	LESTR	CKR-1	CKR-2a	CKR-3	CKR-4	CKR-5	CKR-5
COS-CD4	ADA	798	456	600	816	516	534	153000	3210
	BaL	660	378	600	636	516	618	58800	756
	HxB2	5800	96700	5240	5070	5470	5620	4850	5000
HeLa-CD4	ADA	678	558	4500	912	558	600	310000	6336
	BaL	630	738	1800	654	516	636	104000	750
	HxB2	337000	nd	nd	nd	nd	nd	nd	356000
3T3-CD4	ADA	468	558	450	618	534	606	28400	1220
	BaL	606	738	660	738	534	558	11700	756
	HxB2	456	24800	618	672	732	606	618	606

THIRD SERIES OF EXPERIMENTS

The chemokine SDF-1 (stromal cell-derived factor 1) is the natural ligand for Fusin/CXCR4 and blocks infection by laboratory-adapted strains of HIV-1 (Ref. 1 and 2). SDF-1 exists as at least two forms, SDF-1α and SDF-1ß based on variable splicing of the SDF-1 gene (Ref. 1 and 3) In the RET assay, this chemokine specifically inhibits membrane fusion mediated by gp120/gp41 form the laboratory-adapted strain HIV_{LAI} but not by gp120/gp41 from the macrophage-tropic isolate $HIV\text{-}1_{JR\text{-}FL}$ as shown in Figure 5.

References of the Third Series of Experiments

1. Bleul, C.C., et al. (1996) *Nature* 382:829-833
2. Oberlin, E., et al. (1996) *Nature* 382:833-835
3. Shirozu, M., et al. (1995) *Genomics* 28:495-500

FOURTH SERIES OF EXPERIMENTS

Direct Binding of HIV-1_{JR-FL} gp120 to CCR5$^+$ CD4$^-$ Cells

The direct binding of HIV-1_{JR-FL} gp120 to CCR5$^+$ CD4$^-$ cells has been demonstrated. In this case, preincubation of the gp120 with sCD4 or another CD4-based molecule is required, presumably because this results in a conformational change in gp120 that exposes a chemokine receptor binding site. Figure 6 illustrates the use of flow cytometry to measure the direct binding of sCD4/gp120 complexes to human CCR5-bearing murine L1.2 cells. Background levels of binding were observed with either biotinylated protein alone, or if gp120 from the laboratory-adapted strain HIV-1_{LAI} is used in place of the HIV-1_{JR-FL} gp120 (data not shown).

This assay has been adapted for drug screening purposes to a 96-well microplate format where binding of the sCD4/gp120 complexes to CCR5$^+$/CD4$^-$ cells is measured using a fluorometric plate reader. One method is as follows:

1) Plate out L1.2-CCR5$^+$ cells (approx. 500,000/well).
2) Add inhibitor for 1 hour at room temperature.
3) Wash and add biotinylated sCD4(2.5μg/ml) and biotinylated HIV-1_{JR-FL} gp120 (5μg/ml), then incubate for 2 hours at room temperature.
4) Wash and incubate with streptavidin-phycoerythrin (100ng/nl).
5) Wash and measure the amount of bound gp120/sCD4 using a fluorometric plate reader exciting at 530nm and reading emission at 590nm.

Using this method, inhibition of binding of gp120/sCD4 to CCR5 by CC-chemokines (Fig. 7) and antibodies to CCR5 that block HIV-1 infection (not shown) have been demonstrated.

Inhibition of HIV-1 envelope-mediated membrane fusion by the bicyclam, JM3100.

The bicyclam JM3100, obtained from Dr. J. Moore (Aaron Diamond AIDS Research Center, NY) was tested for ability to inhibit membrane fusion mediated by the envelope glycoproteins of the LAI or JR-FL strains of HIV-1 using the resonance energy transfer (RET) assay described above. As illustrated in Fig. 7, this molecule specifically and potently inhibits fusion mediated by gp120/gp41 from the HIV-1_{LAI} strain, and not from the HIV-1_{JR-FL} strain. These data suggest that this molecule specifically inhibits HIV fusion by blocking the interaction between HIV-1_{LAI} gp120 and CXCR4.

Figure 9. **The CCR5 Binding assay identifies and determines the potency of inhibitors of the gp120-CCR5 interaction.**

HIV-1 Inhibitory monoclonal antibodies were added in a range of concentrations to reecmbinant L1.2 cells that express human CCR5 on their cell surface and used to compete the binding of a complex formed between sCD4 and biotinylated HIV-1_{JR-FL} gp 120, whose binding was detected using a streptavidin-phycoerythrin conjugate. PA-8, -9, -10, -11 and -12 are Progenics' monoclonal antibodies that inhibit HIV-1 entry, while 2D7 is a commercially available (Pharmingen, San Diego, CA) ant-CCR5 monoclonal antibody that inhibits HIV-1 entry. To enhance chemokine receptor expression, both transfected and parental L1.2 cells were treated with sodium butyrate prior to assay (Wu et al., J. Exp. Med. 185:1681).